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A series of expression vectors based on attenuated vaccine strains of Venezuelan equine encephalitis virus (VEE) have been constructed with the ultimate goal of expressing immunizing antigens derived from HIV-1 *in vivo*, thus protecting against subsequent exposure to HIV. To that end we have constructed four such vectors and expressed HIV-1 matrix/capsid, gp160 and gp120 in cell culture and in rodent models. Analogous constructs expressing SIV immunogens also have been generated. These are being evaluated in rodent models in preparations for primate trials.

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INTRODUCTION

We propose to exploit the unique properties of the alphavirus Venezuelan equine encephalitis (VEE) as a vaccine vector for protective immunization against HIV-1. The advantages of the VEE system for delivery of HIV-1 immunogens are as follows:

1. The VEE vaccine vectors are designed for ease of construction and cloning flexibility.
2. VEE vaccine vectors should give high levels of expression and could be modified to express HIV-1 antigens in a variety of intracellular and extracellular forms.
3. The development of these vectors will benefit from the simultaneous refinement of new molecularly cloned VEE vaccine candidates upon which the vaccine vectors will be based.
4. Unlike several of the virus vectors proposed for delivery of heterologous antigens, most of the human population is not already immune to VEE.
5. The VEE glycoproteins will target the vaccine vectors to lymphoid tissue for efficient immunogen delivery.
6. Live, attenuated VEE vaccines induce protection to mucosal challenge. Experiments with a VEE vaccine vector expressing the influenza HA gene demonstrated protection not only against disease following an intranasal influenza challenge but also against infection of the nasal mucosa. Therefore, these vectors induce protection against mucosal challenge in addition to stimulating a strong humoral and cellular response protective against parenteral challenge.

We feel that the advantages offered by the VEE vaccine vectors represent an important opportunity in the search for a safe and effective immunization strategy against HIV-1. The proposed experiments are designed to assess the feasibility of this novel approach.

ANNUAL REPORT BODY

This report will be organized according to the four original objectives: 1) vector development, 2) cloning and cell culture expression of HIV-1 genes, 3) immunological characterization of HIV-1 proteins expressed in cell culture and in animals, and 4) cloning, expression and immunological studies with analogous SIV genes. We have made substantial progress toward each of these objectives.

Objective 1: To construct first generation VEE vaccine vectors containing an additional subgenomic mRNA promoter.

First generation VEE vaccine vectors will be constructed

containing a duplication of the internal promoter which normally drives the production of high levels of a subgenomic mRNA encoding the viral structural proteins. Exogenous sequences will be expressed from the second copy of the subgenomic promoter and will be carried in the genomes of replication competent, attenuated VEE virions. Such vectors will allow straightforward introduction of alternative immunogens for testing.

Two types of vector have been developed, which place the second subgenomic promoter in different portions of the genome (Figure 1). In the downstream position, the second subgenomic promoter and ClaI restriction site were placed between the 3' end of the E1 gene and the beginning of the 3' untranslated region. In the upstream position, the second subgenomic promoter and ClaI site were placed between the 3' end of the nsP4 gene and the authentic 26S subgenomic promoter. Shuttle vectors for both the upstream and downstream vectors were constructed to facilitate the insertion of genes containing internal ClaI sites. The ClaI site is embedded in the 26S mRNAs' untranslated region (leader) between the promoter and the normal AUG start codon for the VEE capsid protein. The genes to be cloned are recovered by PCR using a 5' primer containing a ClaI site, the 3' half of the 26S leader and the capsid AUG such that the inserted gene begins immediately after the capsid AUG. The downstream vector construct expressing the influenza virus hemagglutinin gene has been used successfully to immunize and protect mice against intranasal challenge with influenza virus (Davis et al., 1996).

Upstream and downstream vectors were constructed in two alternative attenuating genetic backgrounds, V3014 (Davis et al., 1991) and V3526 (Davis et al., 1995). V3014 contains two attenuating point mutations, one at E2 209 and the other at E1 272. V3526 contains a lethal deletion of the PE2 cleavage signal in conjunction with a resuscitating and attenuating point mutation at E1 253. Unless otherwise noted, the experiments outlined below were performed with vectors using the V3014 mutant constellation. Upstream and downstream vectors containing attenuating mutations have been used in studies of viability, expression and immunogenicity detailed below.

Objective 2: To characterize cell culture expression of HIV-1 proteins by the VEE vaccine vectors.

Portions of the HIV-1 env and gag genes will be used in initial tests of VEE vaccine vector viability, stability, and expression in cell culture systems. The specific infectivities of transcripts from VEE vaccine vector constructs and the relative size of plaques initiated by the

transcripts will be determined as a measure of viability. The level of expression will be determined by immunocytochemical staining of infected cells, radioimmune precipitation of labelled infected cell extracts, and western blot analysis of unlabelled infected cell lysates. VEE vaccine vectors will be passaged in cell culture, and the genetic stability of the insert and its expression will be assessed. Other immunogens, such as pol, nef, vif, tat, and rev also could be tested.

Three HIV-1 genes have been cloned into the downstream vector: the complete gp160 gene, the gp160 gene truncated following the gp120 sequences, and the MA/CA region of gag with the myristylation site ablated. Transcripts derived from each of these constructs had a specific infectivity on baby hamster kidney (BHK) cells equivalent to transcripts of V3000, the parent clone of the vectors. All three vaccine vector constructs expressed their cognate heterologous gene. For VEE-gp160, expression was demonstrated by western blot of VEE-gp160 infected BHK cell extracts with a gp120 monoclonal antibody. The positive band corresponded with the position of gp120, suggesting that gp160 was processed normally in these cells. The expressed product of VEE-gp120 was predicted to be secreted from infected cells. Synthesis of the product was demonstrated by concentration of tissue culture supernatants and immunoprecipitation of the [^{35}S]-methionine labeled product with the gp120 monoclonal antibody.

Much of our focus has been on the MA/CA constructs. MA/CA expressed from the downstream vector was readily detected by immunoprecipitation or western blot with p17 monoclonal antibody. Moreover, MA/CA was detected as a radioactive gel band when extracts of infected cells, labeled with [^{35}S]-methionine, were analyzed by SDS-PAGE. MA/CA was produced in quantities equivalent to the VEE capsid protein. On Coomassie stained gels of total cell extracts, the amount of MA/CA produced was equivalent to that of prominent cell proteins such as actin.

MA/CA also has been cloned into the upstream vector and its expression demonstrated by immunoprecipitation from extracts of infected BHK cells labelled with [^{35}S]-methionine. Direct comparison of the levels of MA/CA produced from the upstream and downstream vector MA/CA constructs showed that the upstream vector produced approximately one-third the amount of MA/CA produced by the downstream vector. Both upstream and downstream MA/CA vectors were passed 10 times in BHK cells to determine the relative stability of the MA/CA gene in each vector type. As a function of the amount of product made in the first passage, production of MA/CA from the upstream promoter vector was more stable than from the downstream construct. However, on an absolute scale, the

production of MA/CA from the downstream vector remained at a higher level than expression from the upstream vector even after 10 passages.

Objective 3: To test the VEE vaccine vectors for expression of antigenically authentic HIV-1 proteins.

Expression of HIV-1 proteins in an immunologically relevant form will be assessed using HIV-1-positive patient sera for immunocytochemical staining of VEE vaccine vector infected BHK cells, immunoprecipitation of radiolabelled expression products and western blot assays. Mice (BALB/c and transgenics bearing human HLA-A2) will be immunized with VEE vaccine vectors expressing portions of the HIV-1 env gene (gp120) or gag gene. Sera of immunized mice will be tested for anti-HIV-1 antibody by ELISA, by immunocytochemical staining of HIV-1 infected cells, and by HIV-1 neutralization tests. Induction of an HLA-A2 restricted cytotoxic T-lymphocyte (CTL) response will be evaluated following immunization of HLA-A2 transgenic mice. The ability of the VEE vaccine vectors to express human CTL epitopes will be assessed by infecting human cells with VEE vaccine vectors and testing them as targets with Gag- and Env-specific human CTL lines. Immunized mice will be tested for CTL responses using appropriate cells infected with Gag- and Env-expressing vaccinia vectors as targets.

Labelled BHK cell extracts, derived after infection with VEE vaccine vectors expressing MA/CA or gp160, were analyzed by western blot using HIV-1 positive patient sera. These sera reacted strongly with the expressed products, suggesting that at least by this assay, the VEE expressed proteins were antigenically relevant to HIV-1 infections in humans.

BALB/c mice were immunized with 10^4 pfu of the downstream VEE-MA/CA vector inoculated subcutaneously (sc.) into each of their rear footpads. Serum from each of 4 mice, drawn 3 weeks later, reacted with recombinant MA/CA in western blots, and similarly immunized mice were positive by ELISA against bacterially expressed MA/CA (Figure 2). Only a variable proportion of mice inoculated with the upstream VEE-MA/CA vector had a detectable anti-MA/CA titer in ELISA, and where present, the titers were lower than those induced by the comparable downstream vector. When animals primed with the downstream VEE-MA/CA vector were boosted with a total dose of $2x10^5$ pfu of the same construct, each animal demonstrated a boost in titer (Figure 2). Both IgG and IgA were represented in the humoral response. IgA specific for MA/CA also was detected in vaginal washes of these mice. One week after the booster inoculation, 4 mice were sacrificed, and splenocytes were cultured for assay of CTL. Significant CTL activity was detected (Figure 3).

Two experiments have been unsuccessful thus far. We have been unable to detect CTL activity in the HLA-A2 BALB/c transgenic mice. However, these experiments preceded our successful demonstration of CTL activity in normal BALB/c animals. Having now optimized the assay, we plan to return to the transgenic mouse model. A second problem area is with the gp160 construct. Although we can demonstrate strong expression in cell culture, the VEE-gp160 vector did not induce detectable neutralizing antibody in CD-1 mice. We are repeating this experiment in BALB/c mice and are optimizing the neutralization assay.

Objective 4: To test the ability of VEE vaccine vectors expressing analogous simian immunodeficiency virus (SIV) proteins for induction of protective humoral and cellular immune responses in an SIV/primate model.

VEE vaccine vectors will be constructed containing SIV genes analogous to the HIV-1 constructs tested in Objective 3. The ability of vaccinated monkeys to mount a humoral and CTL response to SIV will be assessed. CTL responses will be measured against autologous B cell lines infected with vaccinia viruses expressing SIV proteins. The induction of SIV antibodies will be monitored by ELISA, western blot, and neutralization tests. The VEE vaccine vectors expressing SIV genes will be tested for their efficacy as preventive vaccines, and will be available for further testing as therapeutic vaccines.

The SIV MA/CA and env genes were cloned into the downstream 3014 vector and into the downstream 3526 vector, and the MA/CA gene also was cloned into the upstream 3014 vector. The companion constructs, with the heterologous gene in the reverse orientation, also were generated. Each clone was transcribed *in vitro* and upon electroporation, yielded viable virus. Cells infected with these vectors were labelled with [^{35}S]-methionine, and the cell lysates were analyzed by SDS-PAGE. The appropriate sized bands were apparent with or without prior immunoprecipitation with the cognate antibodies. A comparative immunogenicity study of these constructs in mice is in progress. Renovation of the Children's Hospital Research Foundation primate facility for BL-3 containment of VEE based vectors is nearing completion. The first group of primates has been ordered and will be immunized using a protocol based on the results of the comparative mouse study.

CONCLUSIONS

Substantial progress has been made toward each of the four objectives of the grant. In the coming grant year, we anticipate that the

preliminary evaluation of the SIV constructs in mice will be completed and the initial primate trials with the SIV constructs will have commenced. With the development of SHIV models, it also may be possible to test the HIV constructs for protective efficacy in primate models.

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Davis, Nancy L., Kevin Brown and Robert E. Johnston. 1996. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. J. Virol., in press.

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APPENDIX

1. Figure 1. VEE upstream and downstream double promoter vectors.

2. Figure 2. Serum anti-MA/CA titers from BALB/c mice.

3. Figure 3. Anti-MA/CA CTL induced by VEE vectors.

4. Manuscript: Davis, Nancy L., Kevin Brown and Robert E. Johnston. 1996. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. J. Virol., in press.

Double Promoter Vectors

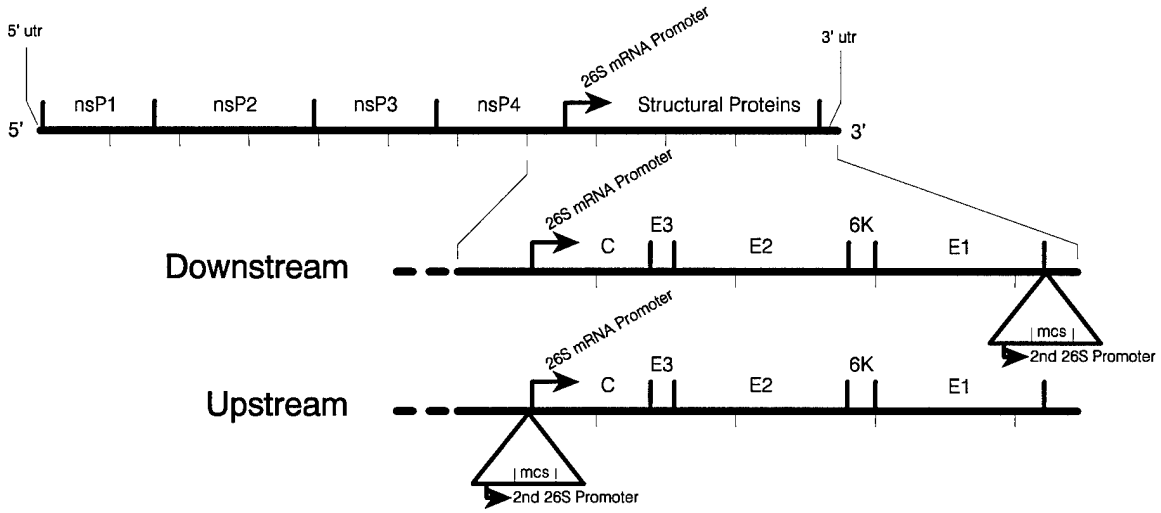

Figure 1.
VEE Upstream and Downstream Double Promoter Vectors

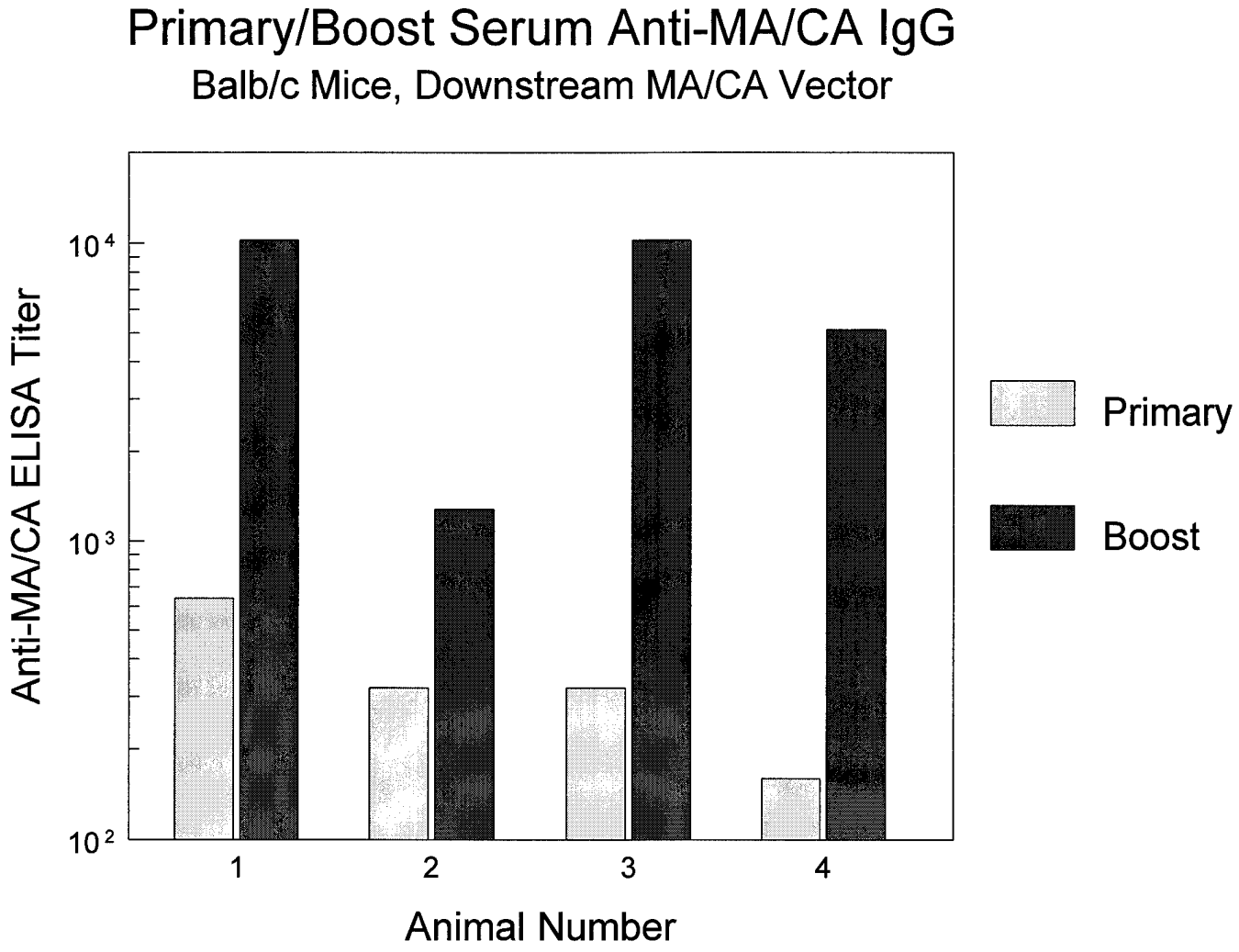

Figure 2. Serum anti-MA/CA IgG titers from Balb/c mice inoculated and boosted s.c. in the rear footpads with the downstream promoter VEE-MA/CA vector. Titers are expressed as the reciprocal of the highest dilution at which the $O.D._{450nm}$ was ≥0.2 after background signal was subtracted.

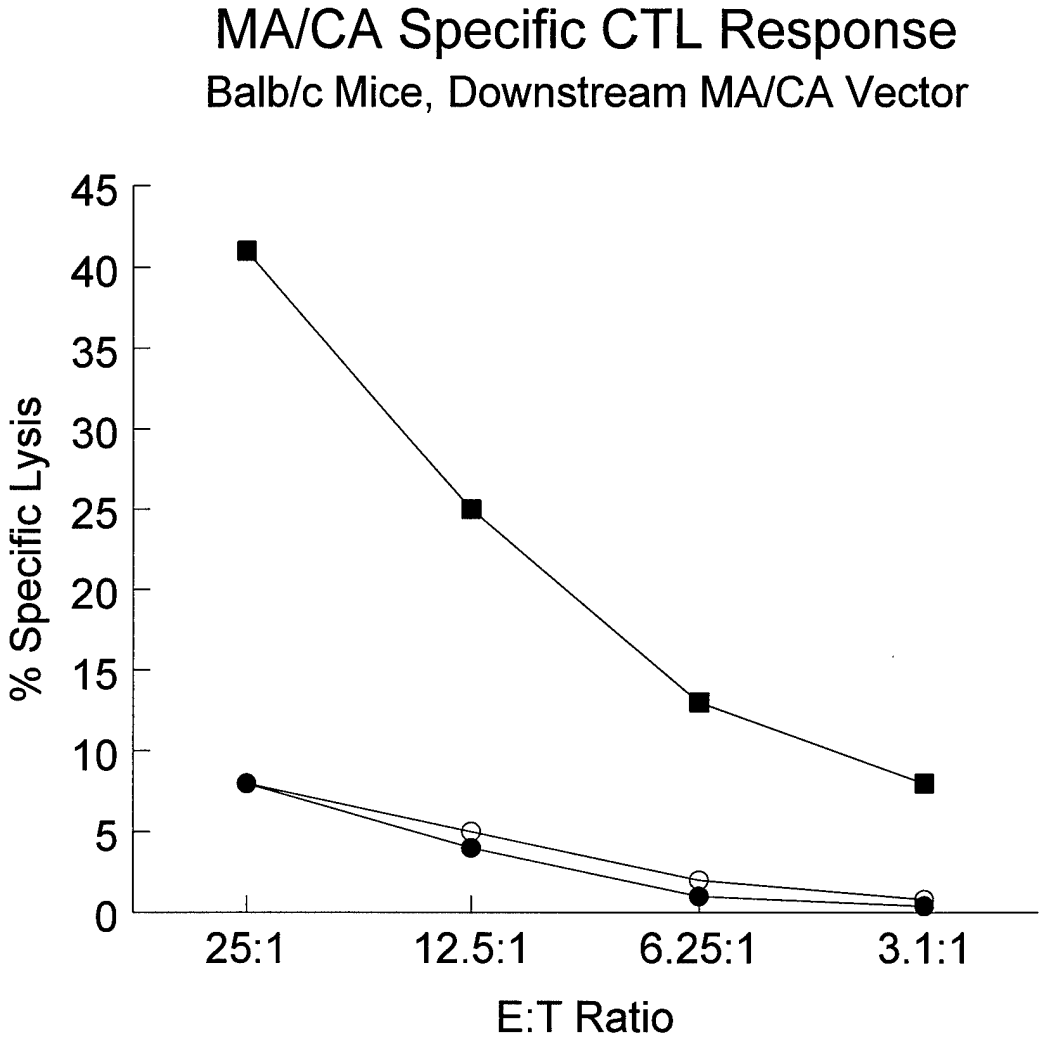

Figure 3. Anti-MA/CA CTL induced by VEE vectors. Splenocytes isolated from Balb/c mice primed and boosted with the downstream VEE-MA/CA vector and stimulated twice *in vitro* specifically lyse vaccinia-Gag infected P815 targets (■), but fail to lyse targets infected with vaccinia-alone (●) or uninfected target cells (○).

A VIRAL VACCINE VECTOR THAT EXPRESSES FOREIGN GENES IN LYMPH NODES AND PROTECTS AGAINST MUCOSAL CHALLENGE

Running title: VEE vaccine vector protects mucosal surface

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ABSTRACT

A candidate live virus vaccine strain of Venezuelan equine encephalitis virus (VEE) was configured as a replication-competent vector for *in vivo* expression of heterologous immunogens. Three features of VEE recommend it for use as a vaccine vector. 1) Most human and animal populations are not already immune to VEE, so that pre-existing immunity to the vector would not limit expression of the heterologous antigen. 2) VEE replicates first in local lymphoid tissue, a site favoring the induction of an effective immune response. And 3) parenteral immunization of rodents and humans with live, attenuated VEE vaccines protects against mucosal challenge, suggesting that VEE vaccine vectors might be used successfully to protect against mucosal pathogens. Upon subcutaneous (sc.) inoculation into the footpad of mice, a VEE vector containing the complete influenza hemagglutinin (HA) gene expressed HA in the draining lymph node and induced anti-HA IgG and IgA serum antibody, the levels of which could be increased by sc. booster inoculation. When immunized mice were challenged intranasally with a virulent strain of influenza virus, replication of challenge virus in their lungs was restricted, and they were completely protected from signs of disease. Significant reduction of influenza virus replication in the nasal epithelia of HA vector-immunized mice suggested an effective immunity at the mucosal surface. VEE vaccine vectors represent an alternative vaccination strategy when killed or subunit vaccines are ineffective or when the use of a live attenuated vaccine might be unsafe.

Vaccination has proved to be the most effective means of controlling viral disease, especially in those cases where live attenuated vaccines, such as those for smallpox, poliomyelitis and measles, have been employed. In these instances, virus mutants replicate within the vaccinated host without causing disease, while inducing protective and long lasting immunity to the naturally occurring pathogenic virus. This approach has been extended by using vaccine virus genomes as vectors to express the genes of other pathogens *in vivo* (2,12,19,25). In this report, we describe the construction of an expression system based on a candidate Venezuelan equine encephalitis virus (VEE) vaccine strain, and its ability to express a heterologous gene both *in vitro* and *in vivo.* The influenza HA gene was used in this study because it afforded a stringent test of mucosal protection in the mouse. Mice vaccinated with the VEE HA vector not only were protected against clinical disease following intranasal influenza challenge, but also exhibited resistance to influenza replication in the lung and at mucosal surfaces of the upper respiratory tract.

VEE is a member of the alphavirus genus, a group which includes Sindbis virus and Semliki Forest virus as prototypes (35). Several properties of alphaviruses have made them useful tools for high level expression of foreign genes both in cell culture and in animals (21,23,24,28,29,37,39). These viruses can infect a wide range of vertebrate and invertebrate cells to initiate a prolific, cytoplasmic replication cycle. Their single-stranded positive sense RNA genomes (between 11,000 and 12,000 nts) are infectious, as are genome replicas derived from

full-length cDNA clones (35). Self-replicating RNA genomes serve as mRNA for an enzyme complex with both RNA replicase and transcriptase activities, and transcription of a subgenomic mRNA for the structural proteins is driven by a very strong, highly conserved promoter sequence. Taking advantage of this high level promoter, alphavirus expression vectors either contain a foreign gene in place of the viral structural protein genes (37), or contain a duplicate subgenomic mRNA promoter that drives expression of a foreign gene (13).

Three biological features of VEE suggest that it could be an unusually effective *in vivo* expression vector for vaccination with heterologous gene products. First, parenteral immunization of rodents and humans with live, attenuated VEE vaccines results in protection not only against parenteral challenge, but also against intranasal (in.) and aerosol challenges (5,7,16,17). This predicts that a delivery system based on a VEE vaccine could induce protection from invasion at mucosal surfaces, which are important routes of entry for many pathogens, including human immunodeficiency virus (HIV). Second, in contrast to most other alphaviruses, VEE replicates first in the lymph nodes draining the site of inoculation (10,11,15), where high level synthesis of a heterologous antigen might result in efficient immunization. Finally, and in contrast to viral vectors based on vaccinia or vaccine strains of adenovirus and poliovirus, most human and animal populations are not already immune to VEE. Therefore, *in vivo* expression of a heterologous immunogen from a VEE-based vaccine vector would not be limited by prior immunity to the vector itself.

MATERIALS AND METHODS

Clones and Viruses.

Plasmid pV3014, a derivative of the full-length cDNA clone of the virulent Trinidad donkey strain of VEE with two attenuating mutations (E2 Lys 209 and E1 Thr 272, described in 8,11), was altered to allow expression of heterologous genes. A duplicate 26S RNA promoter followed by a unique ClaI site was inserted immediately downstream of the E1 gene by oligonucleotide directed mutagenesis of a subclone of the structural protein genes in M13 (18). The promoter sequence and its ability to function in an abnormal context were predicted from previous work with Sindbis virus (13,20). The VEE structural gene region with the auxiliary 26S subgenomic RNA promoter was subcloned into pUC118. The multiple cloning region of the Cla12 adaptor plasmid [(14), the kind gift of S. Hughes, NCI] flanked by ClaI sites, was inserted into the unique ClaI site to form a shuttle vector. The cloned cDNA of the complete HA gene from influenza PR/8/34, type H1N1, kindly supplied by P. Palese, Mt. Sinai School of Medicine, NY, was inserted into the multiple cloning region of the shuttle vector. The HA gene was then transferred using ClaI into the full-length VEE vector clone. Infectious RNA genome equivalents were transcribed *in vitro* from linearized plasmids with T7 RNA polymerase and used in electroporation of baby hamster kidney (BHK) cells to produce stocks of progeny virus (9,22).

An egg-grown stock of influenza strain PR/8/34, generously supplied by H. van Campen, Univ. of Wyoming, Laramie, WY, was used for in. challenge of mice. A similar virus preparation grown from ATCC seed stock VR-95 in embryonated eggs was used for neutralization assays and BHK cell infections.

Immunization and Challenge of Mice.

Adult (four or six-week-old), female CD-1 mice (Charles River) were put under light anesthesia by inhalation of Metofane (Pitman-Moore) and immunized by subcutaneous (sc.) inoculation of 1 x 10^4 pfu of vector without insert or HA vector in a 10 µl volume of diluent [phosphate-buffered saline (PBS) containing 1% donor calf serum (DCS)] into each rear footpad. Mock-immunized controls received diluent alone. Booster inoculations were done three weeks post-immunization, and were identical to the initial immunization, except that the total dose of HA vector or vector alone was 2 x 10^5 pfu. Twenty-one days following the primary immunization, or 18 days following the boost, a challenge dose of 10^5 50% egg infectious doses (EID_{50}) of egg-grown influenza strain PR/8/34 in a total volume of 20 µl PBS was introduced into the nares under light Metofane anesthesia. Under the conditions used here this dose represented one 50% lethal dose (LD_{50}), but induced obvious clinical signs in 100% of inoculated naive animals.

Antibody Assays.

Serum samples were obtained by limited bleeding from tail veins. ELISAs included gradient-purified PR/8/34 influenza virus as antigen and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-mouse IgA as second antibody. Positive controls were the H1-specific IgG2a monoclonal antibody 37-85, produced by W. Gerhard, Wistar Institute, PA (34) and obtained through S. Clarke, UNC, Chapel Hill, and an H1-specific IgA monoclonal antibody kindly supplied by P. Small, University of Florida, Gainesville, FL. In these ELISAs, an $O.D._{450}$ greater than 0.2 above background (wells without serum) was scored as positive. Pre-immunization mouse sera and sera from PBS or vector alone inoculated control mice showed variable reactivity at a 1:50 dilution in the influenza ELISA, possibly caused by nonspecific binding of sialylated serum immunoglobulins to the influenza hemagglutinin. Therefore, positive reactivity at dilutions of less than 1:100 were considered background. Plaque-reduction neutralization assays were carried out essentially as described in Olmsted, *et al.* (27). Virus was incubated for 30 min. at 37C with dilutions of serum in the presence of 5% guinea pig serum (Cedarlane) as a source of complement, followed by a second 30 min. incubation at 37C with a 1:200 dilution of goat antiserum to mouse immunoglobulin (Cappel). Standard plaque assay of serum-treated virus was done on Madin Darby canine kidney (MDCK) cells overlayed with agarose without serum, but containing 1 μg/ml trypsin. Titers represent the highest dilution that reduced plaque number by 60%.

***In Situ* Hybridization Analysis.**

Tissues were prepared for *in situ* hybridization as described (6,11). Popliteal lymph nodes were taken at 24 hr after sc. inoculation with VEE vector, while heads were processed at forty-eight hours after in. challenge with influenza virus, following perfusion with 4% paraformaldehyde in PBS. Similarly sized, ^{35}S-UTP-labeled, negative-sense riboprobes were prepared from transcription vectors containing either the identical HA gene used in the construction of the VEE HA vector, a subclone of the VEE structural genes or a subclone of the Epstein-Barr virus genome obtained from N. Raab-Traub, UNC, Chapel Hill. The lengths of the respiratory epithelial surface in coronal sections incubated with the HA-specific riboprobe, both total length and that associated with positive *in situ* signal, were measured using a Nikon FXA microscope and a Macintosh Quadra 840 AV computer with the public domain NIH Image program (developed at the NIH and available from the Internet by anonymous FTP, zippy.nimh.nih.gov, or floppy disk from National Technical Information Service, Springfield, VA, PB95-500195GEI).

RESULTS

Construction of an Attenuated VEE Expression Vector Containing the Influenza HA Gene.

A full-length cDNA clone of the VEE RNA genome was the basis for construction of a panel of live, attenuated VEE vaccine candidates (8). A representative vaccine

candidate, V3014, contains two attenuating mutations (E2 Lys 209 and E1 Thr 272), is avirulent following sc. inoculation of adult mice, and grows to high titers in the draining lymph node without extensive pathology (8,11). V3014 was converted to a replication competent VEE vaccine vector by placing a second copy of the 26S subgenomic RNA promoter and a unique ClaI site at the exact 3'-end of the structural gene region. To test this vector for its ability to express a heterologous gene, the cloned influenza hemagglutinin (HA) gene from strain PR/8/34 was introduced at the unique ClaI site downstream of the second subgenomic promoter. The influenza HA protein is well-characterized, as is the disease course following in. inoculation of mice with strain PR/8/34 (38). Thus, this system provides 1) a mouse model for direct assessment of the ability of the VEE vaccine vector to induce an immune response not only to VEE proteins, but also to a heterologous protein, and 2) a challenge model in which to test the capacity of that response to protect against influenza challenge across a mucosal surface.

VEE vector virus without an insert, HA vector virus and AH vector virus (containing the HA gene in the noncoding orientation) were produced by transfection of BHK cells with *in vitro* RNA transcripts of the appropriate cDNA clones. Immunoprecipitation and immunocytochemistry were used to show that authentic HA protein was synthesized in BHK cells infected by the HA vector. HA vector-infected cells, but not cells infected with AH vector or vector alone, contained a protein that was precipitated by anti-influenza rabbit polyclonal antibody (obtained from R. Webster and B. Meyer, St. Jude Medical Center, Memphis TN) and

that migrated on SDS-containing polyacrylamide gels similarly to HA protein precipitated from BHK cells infected with influenza virus (data not shown). Although high levels of HA were produced in HA vector-infected cells, VEE virions produced by these cells contained no detectable HA protein (data not shown). Immunocytochemical staining with an HA-specific monoclonal antibody (34) demonstrated that the amount and intracellular location of HA were similar for HA vector-infected BHK cells and influenza virus-infected cells (data not shown). Cells infected with VEE vector alone did not bind the HA specific monoclonal antibody. About one-fourth of the HA vector-infected cells (estimated from the proportion of cells reacting with anti-VEE antibody) did not react with the anti-HA monoclonal antibody. This may reflect a lower detection limit for the polyclonal anti-VEE antibody than for the HA monoclonal antibody, or indicate that the HA gene was not expressed in all HA vector-infected cells. The possibility that in some cells the large HA gene (1.7 kb) was deleted from the VEE vector during vector replication is suggested by results with some large inserts in the analogous Sindbis virus vector system (13).

Expression of HA in Draining Lymph Nodes of HA Vector-Inoculated Mice.

The major site of replication of the parent attenuated virus, V3014, in CD-1 mice inoculated in the rear footpad is the draining popliteal lymph node (11). V3014 does not cause significant tissue destruction in the lymph node, although it replicates to titers equivalent to those of its virulent progenitor within 24 hrs pi. To determine whether the VEE double promoter

vector replicated and expressed a heterologous gene at this site, popliteal lymph nodes taken from mice 24 hrs after a primary inoculation in the rear footpads with 2 x 10^4 pfu of either vector alone or the HA vector were analyzed for the presence of virus-specific RNA by *in situ* hybridization. The lymph node from the HA vector-inoculated mouse bound both the VEE and HA-specific negative-sense riboprobes, whereas the tissue from animals inoculated with vector alone bound only the VEE riboprobe (Fig. 1). No signal was detected in any of the negative controls, which included similar sections from these lymph nodes incubated with an irrelevant, similarly sized riboprobe containing Epstein-Barr virus sequences and lymph node sections from mock-inoculated mice incubated with all of the riboprobes. *In situ* hybridization analysis of spleen and decalcified legs taken at 24 hrs post inoculation with the HA vector also showed specific hybridization with both the HA and VEE riboprobes, while parallel preparations from mice inoculated with vector alone reacted only with the VEE riboprobe (not shown). Therefore, as predicted from the phenotype of the attenuated VEE parent, the VEE HA vector replicated at the site of inoculation, in the draining lymph node and to a lesser degree in the spleen, and expressed the inserted HA gene at all of these sites.

Anti-HA Antibody Levels in Sera of VEE Vector-Immunized Mice.

A correlation between the presence of anti-influenza serum antibody and protection of mice against influenza-induced pneumonitis and death has been shown previously (for example, see reference 31). The delivery of HA to the lymphoid tissues of the HA vector-

immunized mice was effective in eliciting a measurable humoral immune response to HA. Groups of 12 six-week-old mice were given a primary immunization by sc. inoculation of 2×10^4 pfu of vector alone or HA vector or an equal volume of diluent into the rear footpads, and three weeks later were given sc. booster inoculations. The boosters contained, in the case of both vector- and HA vector-immunized mice, ten times the original dose of virus, or 2×10^5 pfu. Pre-immunization sera, sera collected three weeks following the primary immunization, and sera collected 12 days after the booster immunization were tested by ELISA for anti-HA serum IgG and IgA. After a single inoculation 75% of the HA vector-inoculated mice had positive anti-influenza serum IgG titers, and 25% had positive anti-influenza serum IgA titers (Table 1). The booster inoculation increased these titers, and gave 100% positive response for IgG and 75% for IgA. Inoculation with vector alone or HA vector induced a strong primary anti-VEE humoral antibody response (Table 1). Although the vigorous anti-VEE immune response probably limited the replication of the booster inoculum, sufficient HA protein was expressed from the second inoculum of HA vector to increase the anti-influenza titers substantially (Table 1).

Serum samples taken 12 days after the booster immunization also were tested for neutralizing activity against the PR/8/34 strain of influenza virus in a plaque reduction assay. Sera from mice in the two control groups (those inoculated with diluent or with vector alone) did not neutralize influenza virus at a dilution of 1:20, the lowest tested. However, all sera from the HA vector-immunized mice showed neutralizing activity. Ten of the 12 sera from this group

gave a geometric mean 60% plaque reduction titer of 1175, while samples from two of the 12 mice showed 30% and 56% plaque reduction at the lowest dilution. As a benchmark comparison, convalescent sera (post-challenge sera from four surviving mock-immunized mice) gave a geometric mean 60% plaque reduction titer of 460.

Protection of HA Vector-Immunized Mice Against Intranasal Challenge.

Protection from clinical disease.

Three weeks after receiving a single sc. dose of diluent, 2×10^4 pfu of vector alone or 2×10^4 pfu of HA vector, mice were challenged in. with influenza strain PR/8/34, a strain that induces a lethal pneumonitis in mice infected by this route (38). Results recorded over a two week observation period showed that inoculation with the HA vector conferred significant protection against mortality ($p < 0.01$) and morbidity ($p < 10^{-4}$). Mice in the two control groups suffered 54% mortality overall, and all the survivors showed moderate to severe signs of disease for an average of 5.1 days ± 1.9. In contrast, 7 of 12 immunized animals showed no signs of infection, and 3 other animals showed very mild signs for an average of 2.3 days ± 1.2. One HA vector-immunized mouse died and one showed severe signs for one day prior to recovery. These two individuals had undetectable anti-influenza serum IgG prior to challenge.

In a separate experiment, three groups of 12 mice received both a primary and booster immunization of either vector alone or HA vector, or were mock immunized with diluent

(antibody titers in Table 1). At 18 days following the boost these mice were challenged in. with influenza strain PR/8/34. Four mice in each group were sacrificed at 48 hr post challenge for *in situ* hybridization analysis of nasal tissue (see below). For the remaining mice, results recorded over a 22-day observation period showed that immunization with the HA vector conferred complete protection against mortality and morbidity (Fig. 2). Mice in the two control groups suffered 44% mortality overall, and all the survivors showed clinical signs of disease for an average of 4.3 ± 1.7 days. In contrast, none of the HA vector-immunized animals died or showed any clinical signs of disease.

Protection of HA vector-immunized mice against illness also was indicated by individual daily weight measurements. Mice receiving two inoculations of diluent or vector alone showed an average maximum weight loss (percent of initial weight) of 23.4 ± 5.6% and 26.0 ± 11.2%, respectively, while mice immunized twice with HA vector lost only 8.4 ± 5.0%. By 9 days post challenge, HA vector-immunized mice on average had recovered their initial weight, while surviving mice that received diluent or vector alone still weighed an average of 19.5 ± 6.2% and 21.7 ± 6.2% less than their initial weights, respectively.

As would be predicted from previous results with V3014 and other attenuated VEE strains (7,8,16,17), mice inoculated with a single sc. dose of 2×10^4 pfu of vector alone or HA vector were completely protected against VEE-induced disease following either ip. or in.

lethal challenge with 10^4 pfu of virulent VEE (data not shown).

Restriction of challenge virus replication in vivo.

Parenteral immunization with the HA vector also reduced the level of influenza virus replication in the lung (Fig. 3). Mice received a single sc. inoculation of vector alone, HA vector or diluent, and were challenged in. with virulent influenza three weeks later. At four days post challenge, no influenza virus was detected in the lungs of 12 HA vector-immunized mice. Eight of 12 diluent controls and 5/12 vector alone controls were positive for influenza replication in the lungs, and had geometric mean titers of 3.0 and 1.9×10^6 pfu/gm, respectively.

Replication of the challenge virus in the nasal epithelium was measured directly using *in situ* hybridization with an HA-specific riboprobe. Mice were immunized with primary and booster doses of vector alone or HA vector, or mock immunized, and challenged in. with virulent influenza virus as described above. Forty-eight hrs after challenge, the animals were sacrificed, and the heads prepared and sectioned for *in situ* hybridization (6). Four mice were selected from each group. Using the serum neutralizing antibody level as a general indicator of the overall HA-specific response, HA vector-immunized mice with different intensities of response were chosen. One *in situ* sample was from the HA vector-immunized mouse with the lowest level of serum neutralizing antibody and minimal reactivity in ELISA, while the other 3 mice had 60% plaque reduction neutralizing titers of 500, 1000, and 5000. Intense confluent

stretches of positive *in situ* signal marked the epithelial surface of the upper respiratory tract in sections from mice inoculated with vector alone or diluent (Fig. 4A), indicating vigorous replication and cell to cell spread of the challenge virus. In contrast, sections from HA vector immunized mice showed fewer and smaller foci of positive *in situ* signal (Fig. 4B), indicating restriction of replication and limitation of spread of the challenge virus.

A morphometric analysis of the coronal sections used for *in situ* hybridization was performed to compare the percentage of the upper respiratory epithelial surface that bound the HA riboprobe among animals in the three groups. The mean percentage for the combined control groups was 16.21 ± 5.43%. This value and the percentages for individual HA vector-immunized mice were compared with respect to their corresponding serum neutralizing antibody titers (Fig. 4C). The upper respiratory tract epithelium from the HA vector-immunized mouse with the lowest neutralizing antibody titer (<20) was among the least resistant to replication of the influenza challenge virus, while sections from the three mice with strong anti-HA immune responses showed a significant reduction in the extent of challenge virus replication (mean percentage of 3.65 ± 3.12%). Therefore, successful immunization with the HA vector, indicated by high levels of anti-HA serum neutralizing antibody, conferred significant protection against replication of virulent influenza virus in the target cells of the upper respiratory mucosa. In a separate experiment with mice immunized only once with vector alone or HA vector, the combined percentage of upper respiratory epithelial involvement was reduced by greater than

50% in the HA vector-immunized mice (data not shown). These results show that the protective effect of a parenteral immunization with the HA vector extended to a vulnerable mucosal surface. The immunological mediators of that protection remain to be determined, but are presumed to include HA-specific antibody at the mucosal surface.

DISCUSSION

The properties of VEE vaccine strains in humans and in rodent models predicted two characteristics of a VEE vaccine vector expressing a heterologous gene. The first prediction was that a heterologous gene would be expressed in the lymph node draining the site of inoculation, based on the finding that the parental VEE strain and its attenuated mutant, V3014, replicate extensively at this site. In work presented here, we showed that the HA vector, derived from V3014, also replicated and expressed the HA gene in lymph nodes, a site expected to favor the efficient induction of an immune response. In fact, the average level of anti-influenza serum neutralizing antibody induced by the VEE HA vector was in excess of that found among control animals that survived influenza-induced disease.

The second prediction was that protection against challenge at a mucosal surface would be evident even when the VEE vaccine was administered parenterally. Live attenuated VEE vaccines can induce protection against intranasal or aerosol challenge regardless of the

route of administration. Protection against disease caused by virus invasion across a mucosal surface may occur either at the level of the mucosal surface itself, by preventing the initial infection of epithelial target cells, or at a later step, by preventing spread of the virus and the resulting clinical signs. In work not presented here, sc. immunization of mice with V3014 induced a vigorous humoral antibody response, substantial anti-VEE IgA on the vaginal surface, detectable anti-VEE IgA in nasal secretions, and complete protection of the nasal neuroepithelium from infection following in. challenge with virulent VEE virus (5). This represents a case in which specific antibodies are detected both in the serum and at the mucosa, and strong protective immunity is observed at the level of the mucosal surface. Likewise, we have now demonstrated that immunization with the HA vector derived from V3014 provided significant protection at the level of the nasal mucosa upon in. influenza challenge. When effective protection is observed at the level of the mucosal surface, it may involve one or more distinct elements, including secretory IgA antibody, transudated humoral IgA and/or IgG antibodies, or a specific cellular immune response (reviewed in 26). Although the exact contribution of each element of the immune response to this protection remains to be determined, we have detected low levels of HA specific IgA in vaginal wash samples from mice immunized by sc. inoculation with the HA vector (unpublished results).

As might be expected for a virus with a highly structured icosahedral capsid (35), there is a limit to the amount of heterologous genetic material that can be stably accommodated

in the alphavirus genome. Our results suggest that a proportion of HA vector viruses loses the property of HA expression during successive rounds of replication in cultured cells. However, other inserted genes, not described here, are more stably expressed from this vector (4). It appears that multiple factors, including, but not limited to, the length of the heterologous sequence, determine stability of expression. The HA protein may be unusually detrimental to VEE replication, which increases the selective pressure for its deletion. Therefore, results using the HA gene may represent a minimal estimate of the immunizing efficacy of VEE vaccine vectors.

The influenza challenge model in mice was used here as a feasibility test for protective immunization with replication-competent VEE vaccine vectors. There is good potential for human use of an attenuated VEE vaccine configured as a vaccine vector based on long experience with an investigational live attenuated VEE vaccine (TC-83), the only such alphavirus vaccine tested extensively in humans (3). More recent recombinant live attenuated VEE vaccine candidates are superior to TC-83 with respect to both safety and efficacy in rodents (8), horses (1) and primates (30). Unintentional spread of an engineered VEE strain by the mosquito vector, an important safety issue, is made extremely unlikely in that V3014, the attenuated parent of the vector described here, does not produce a serum viremia in rodents (11), and even the virulent Trinidad donkey strain of VEE produces a viremia in humans too low to support transmission by mosquitoes (33). Other attenuating mutations under consideration

appear to reduce specifically the efficiency of transmission by the mosquito vector (36).

Engineering live virus vaccines for immunization against heterologous antigens is an active area of experimentation. To this end, expression of foreign genes has been accomplished with members of several virus families, eg. poxvirus, adenovirus, picornavirus, herpesvirus, and orthomyxoviruses, as well as alphaviruses (reviewed in 32). In fact, it is very unlikely that one vector system will be optimal in all respects, such as the length of heterologous sequence that can be maintained stably in the vector, level of efficacy, induction of mucosal protection, potential problems associated with pre-existing immunity to the vector and issues of safety, such as the potential for latent infection. Moreover, the generation of a strong immune response to the vector itself will allow only a limited number of applications of a specific vaccine vector in a given population. Therefore, efforts to produce an ideal vector for all applications are likely to be unproductive. Rather, a strong case can be made for parallel development of multiple virus vaccine vectors, including VEE, which will make available the unique advantages of each for specific applications.

In summary, we have described the generation of a replication-competent VEE expression vector capable of driving the efficient synthesis of a heterologous protein in cell culture and *in vivo*. This vector replicated in lymphoid tissue draining the sc. inoculation site, where it expressed an inserted influenza HA gene. The resulting immune response was

characterized by high levels of anti-HA serum antibody. Following in. challenge with virulent influenza virus, we observed complete protection against clinical signs of disease, restriction of challenge virus replication in the lungs, and significant protection of the nasal mucosal surfaces. These findings suggest that a new generation of rationally engineered, molecularly cloned live VEE vaccines may serve as effective and safe vaccine vectors, protecting against systemic and mucosal pathogens for which other vaccine strategies are not effective or feasible.

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TABLE 1

Serum Antibody Titers in HA Vector-immunized Mice[a]

	Anti-HA		Anti-VEE[b]	
	IgG	IgA	IgG	IgA
Post primary	270	126	16,000	6600
Post boost	562	467	25,400	8980

[a]Geometric mean titers for samples giving an $O.D._{450}$ ≥0.2 at dilutions of 1:100 or greater. Pre-immunization titers were consistently <100. Of 24 animals in the control groups, 22 had anti-HA serum IgG and IgA titers <100, one had an IgG titer of 100 before but not after the boost, and one had an IgA titer of 100 following the boost.

[b]Mean anti-VEE titers for mice immunized with HA vector (shown here) or vector alone (not shown) were not significantly different from each other (Student's t-test, p values ranged from 0.11 to 0.94).

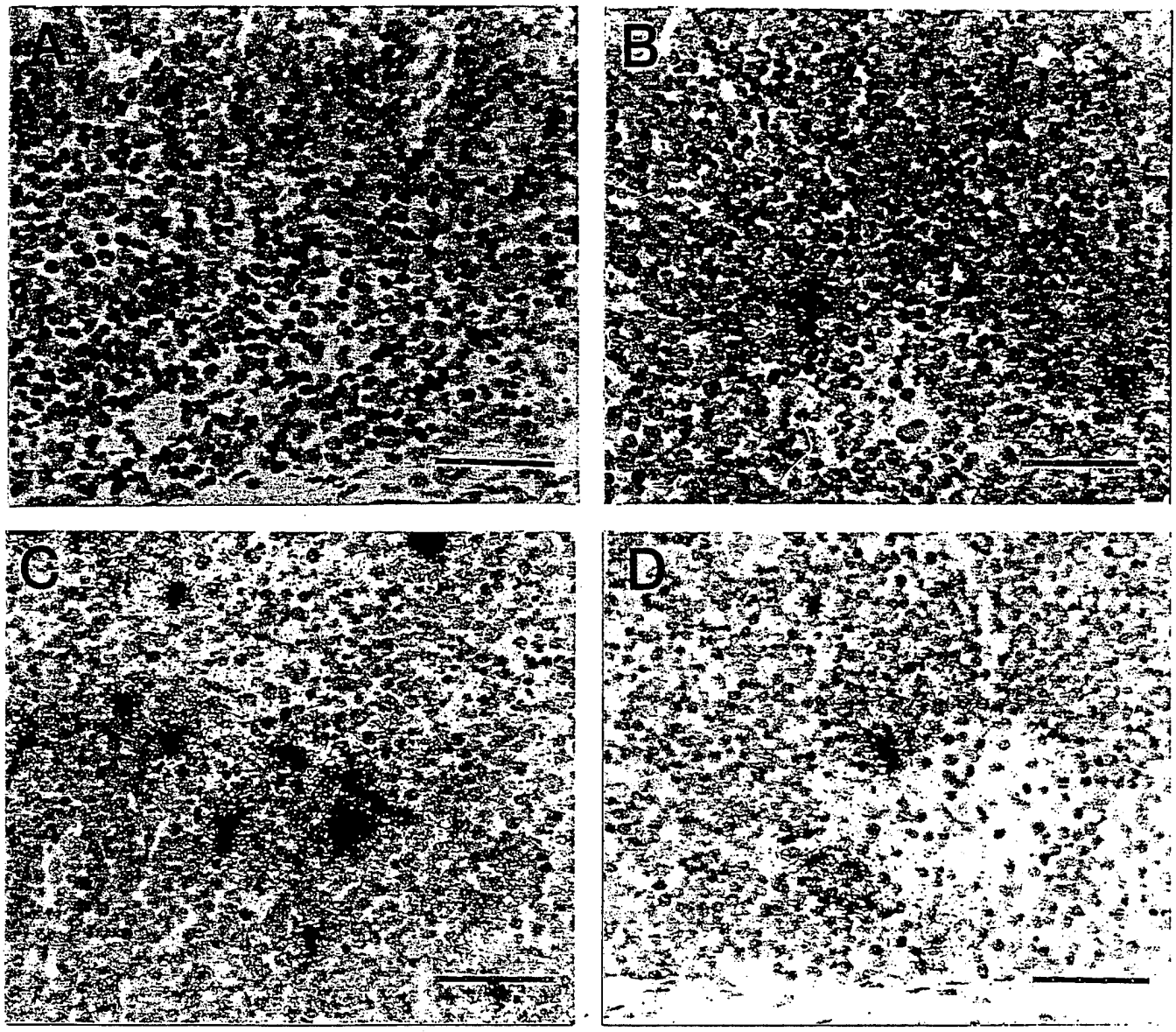


Figure 1. Detection of coding-sense HA RNA sequences in the popliteal lymph nodes of mice inoculated with the HA vector. Popliteal lymph nodes from mice inoculated with vector alone (A and C) or HA vector (B and D) were harvested 24 hr post inoculation and processed for *in situ* hybridization. Similar sections were probed with an ^{35}S-labeled Haspecific riboprobe (A and B) or an ^{35}S-labeled VEE-specific riboprobe (C and D). Magnification is 350x and bar equals 50 *u*m.

Figure 2. Protection resulting from primary and booster immunizations with VEE HA vector against intranasal challenge with virulent influenza virus. Mice received primary and booster inoculations of diluent (Panel A), vector alone (Panel B) or HA vector (Panel C), were challenged intranasally with influenza PR/8/34 at 18 days following the boost and were observed for 22 days. Clinically normal mice are designated by clear bars, and deaths by solid black bars. Mildly affected mice showed slight ruffling (hatched bars). Moderately ill mice showed ruffling, hunching and slowed movement (loose crosshatch). Severely ill mice showed lack of movement, obvious wasting and labored breathing (dense crosshatch).

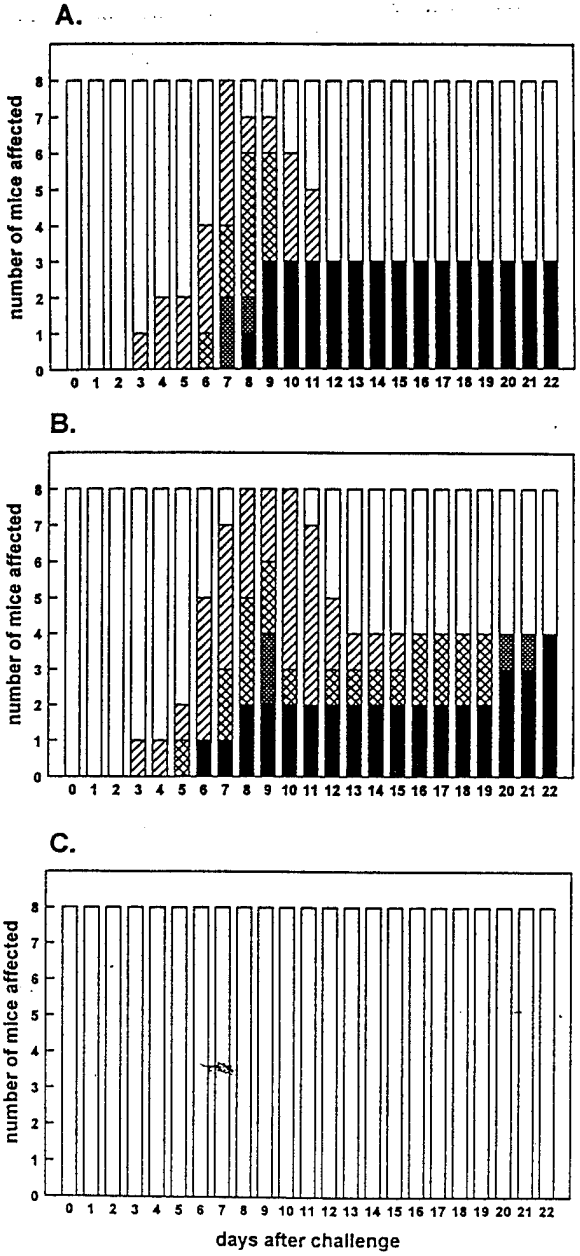
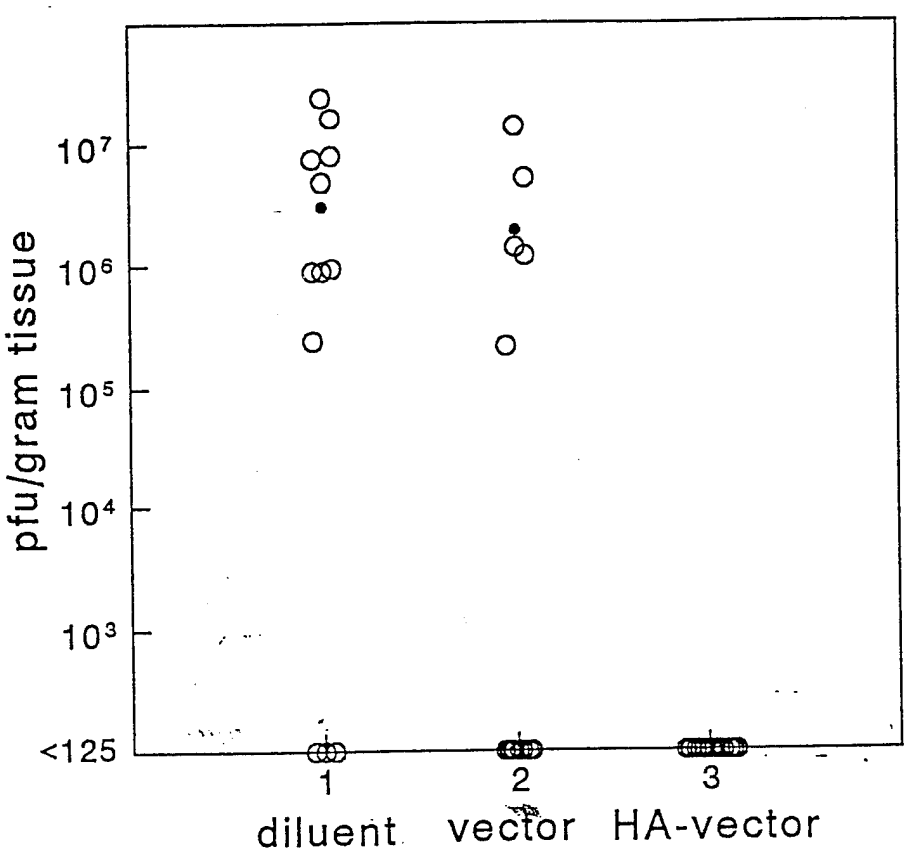


Figure 3. Restriction of influenza virus replication in lungs of HA vector immunized mice. Mice were inoculated sc. with diluent, vector alone or HA vector, and 26 days later were challenged intranasally with influenza PR/8/34. Mice were euthanized on day 4 post-challenge and lungs were homogenized in PBS with 0.s% (w/v) bovine serum albumin to give a 20% (w/v) suspension, clarified by centrifugation and stored at -70C. A standard plaque assay on MDCK cells was performed with duplicate aliquots. The limit of detection was approximately 25 pfu per average lung. Geometric mean titers (solid dots) were calculated for control mice with measurable virus titers.



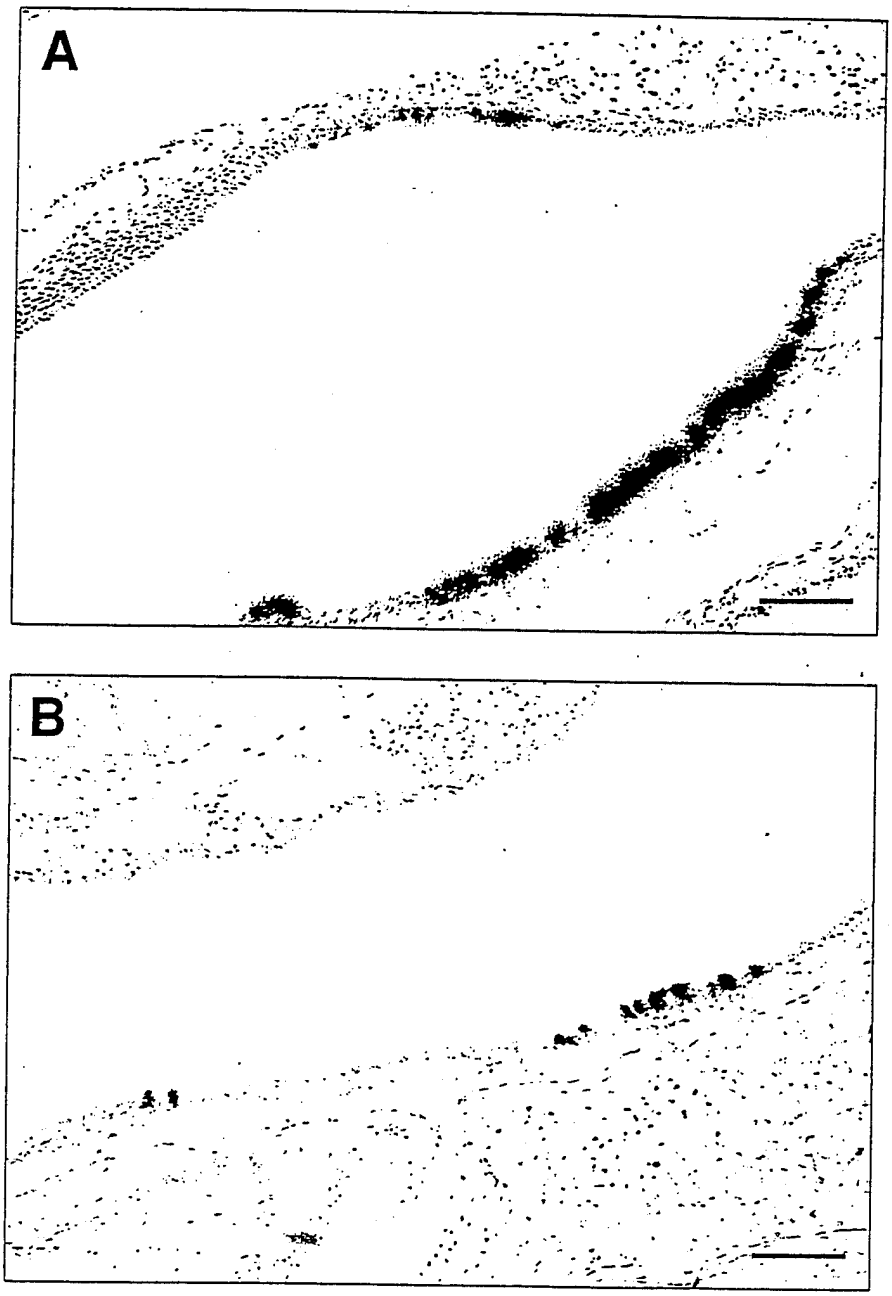


Figure 4 A and B

Detection of HA mRNA sequences in the nasal epithelia of mice challenged intranasally with virulent influenza virus. Mice were immunized and challenged as described in Figure 4. Forty-eight hours post-challenge, heads were processed for *in situ* hybridization. Coronal sections from mice inoculated with diluent (A) or HA vector (B) were incubated with an S-labeled, negative-sense HA-specific riboprobe. The percent respiratory surface involved for the HA vector immunized mouse (B) was 4.7%, similar to the average of 3.65% for mice with a strong anti-HA immune response. No signal was detected in similar sections incubated with an VEE virus-specific riboprobe. Magnification is 123x and bar equals 100*u*m.

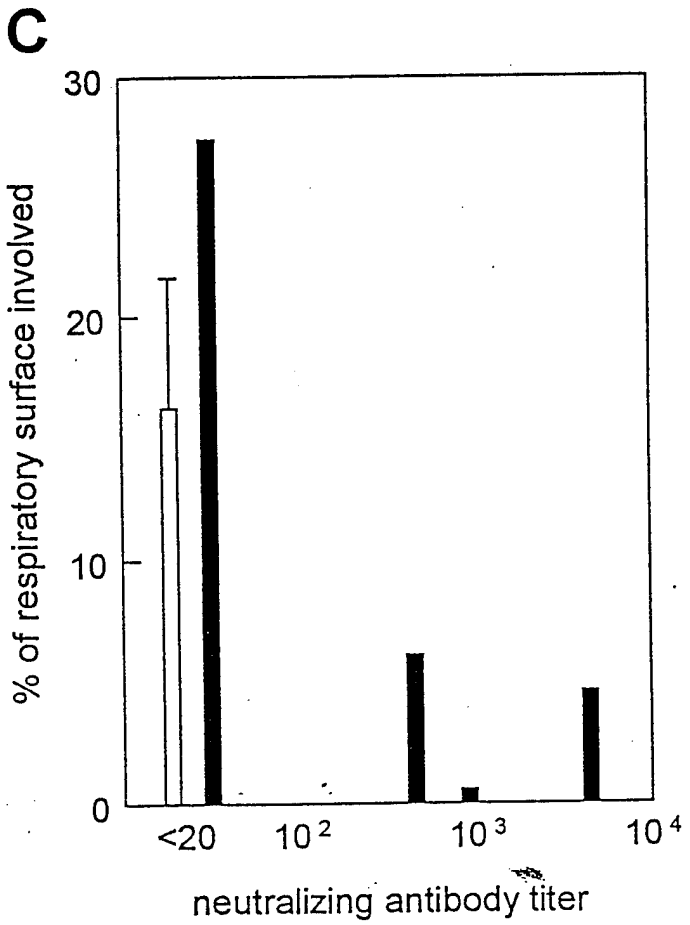


Figure 4 C
(C) Percent respiratory surface involved (the length of respiratory surface in each section showing positive *in situ* signal divided by the total length x 100)was calculated for individual HA vector-immunized mice (black bars), averaged for controls (white bar) and plotted relative to titers of anti-influenza neutralizing antibody.